

**Repellent and mosquitocidal effects of leaf extracts of *Clausena anisata* against the *Aedes aegypti* mosquito (Diptera: Culicidae)**

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## Abstract

Mosquitoes are rapidly developing resistance to insecticides that millions of people relied on to protect themselves from the diseases they carry, thereby creating a need to develop new insecticides. *Clausena anisata* is used traditionally as an insect repellent by various communities in Africa and Asia. For this study, the repellency and adulticidal activities of leaf extracts and compounds isolated from this plant species were evaluated against the yellow fever mosquito, *Aedes aegypti*. In the topical application assays, using total bites as an indicator, repellency was dose-dependent, with the acetone crude extract (15% ) having 93% repellence and the hexane fraction (7.5%) 67% repellence after 3 h. Fractionation resulted in a loss of total repellence. As mosquito-net treating agents, the acetone and hexane extracts of *C. anisata*, both at 15%, had average repellences of  $46.89 \pm 2.95$  and  $50.13 \pm 2.02$  %, respectively, 3 h after exposure. The *C. anisata* acetone extract and its hexane fraction caused mosquito knockdown and eventually death when nebulised into the testing chamber, with an  $EC_{50}$  of 78.9mg/ml (7.89%) and 71.6 mg/ml (7.16%) in the first 15 minutes after spraying. *Clausena anisata* leaf extracts have potential to be included in protection products against mosquitoes due to the repellent and cidal compounds contained therein.

Keywords: botanical repellents; vector control; yellow fever mosquito

## ***1 Introduction***

Vector-borne diseases are a global problem and are increasing in prevalence due to climate changes (Githeko et al. 2000; Colon-Gonzalez et al. 2013). Mosquitoes are by far the most important of the disease vectors and transmit over ten human and/or animal diseases which include malaria, yellow fever, dengue fever, West Nile virus, rift valley fever, chikungunya, Japanese encephalitis, Venezuelan equine encephalitis and Murray Valley Encephalitis amongst others. Vector control has long been seen as a major tool in the control of these diseases. However, effective mosquito control is a complex and difficult problem, as illustrated by the continuing prevalence (and spread) of mosquito-transmitted diseases. Chemical insecticides remain the first line of defence against diseases spread by mosquitoes; however resistance to commonly used insecticides is on the rise, having been detected in 64 countries (WHO 2012). At present there are only four approved classes of insecticides, namely organophosphates, pyrethroids, carbamates organochlorines, with only two modes of action. This limited number in conjunction with a paucity of approvals for new insecticides for vector control by WHO in the last 30 years, has greatly increased the threat of resistance and reduced the sustainability of chemical insecticides for vector control (David et al. 2013). Despite all this, targeting the vector remains the most effective option to significantly reduce disease transmission at this stage. Thus new active ingredients based on novel modes of action or insecticidal compounds acting on new binding sites in already established targets are required to delay the onset of resistance to available insecticides.

Plants are an invaluable potential source of new repellent agents due to the large number of insecticidal compounds found in plants as defences against insects (Furstenberg-Hagg et al. 2013). Most plants naturally produce compounds that are useful in preventing attack from plant-eating insects. These compounds may be classified as repellents, feeding deterrents,

toxins or growth regulators. These compounds are, however, also effective against mosquitoes and other biting Diptera especially the volatile components that are released to deter herbivores (Pichersky and Gershenzon 2002). Efforts to evaluate and develop plant-based repellents have become far more rigorous in recent years. Maia and Moore (2011) provide an extensive list of plants that have been evaluated for efficacy as mosquito repellents.

*Clausena anisata* (Wild) Hook. f. ex. Benth is widely used against various pests and parasites in many parts of Africa. It is used as a mosquito repellent in some parts of South Africa (Mavundza et al. 2011). In Zimbabwe the leaves are used to expel maggots from wounds of animals (Chavunduka 1976). In Cameroon it is used as an insecticide against stored-grain pests. The insecticidal and repellent activities against stored grain pests and feeding-deterrence activity against blowfly larvae have been confirmed in scientific studies (Boeke et al. 2004; Ndomo et al. 2008; Mukandiwa et al. 2013). In Kenya and Ethiopia, the leaves of *C. anisata* are used against intestinal worms (Muthee et al. 2011; Firaol et al. 2013). *Clausena anisata* leaves are densely dotted with glands that produce a strong scent when damaged as in herbivory. The scent is highly unpleasant, characteristic of horse urine as suggested by the common Afrikaans name, *Perdepis* (Horse urine) (Schmidt et al. 2002). The plant occurs from Guinea and Sierra Leone eastwards to Ethiopia, the Sudan and southward to the Cape Province, only avoiding the driest regions. It also occurs in tropical Asia and South-East Asia. It is cultivated in Malaysia and Indonesia (Tchinda 2011).

In this study, we sought to determine the activity of extracts of *C. anisata* against *A. aegypti* when applied topically onto guinea pigs and sprayed on a net; and when used as an aerosol spray. Also included in this study was the pure compound seselin previously isolated and

identified from *C. anisata* (Mukandiwa et al. 2013). The *Aedes aegypti* mosquito is a vector of various diseases in humans and livestock. It is the principal vector of viruses that pose a threat to human health such as dengue, chikungunya, and yellow fever viruses (Gubler 1998). Dengue fever is regarded globally as the most important arthropod-borne viral disease. It is endemic in at least 100 countries in Asia, the Pacific, the Americas, Africa, and the Caribbean, occurring every year during a season when *Aedes* mosquito populations are high. About 50% of the world's population lives in areas where there is a risk of dengue transmission (Murray et al. 2013). The *Aedes aegypti* mosquito (Diptera: Culicidae) has also been implicated in the mechanical transmission of various diseases in livestock such as Rift Valley fever (Hoch et al., 1985), lumpy skin (Chihota et al. 2001) and anthrax (Turell and Knudson 1987). All these diseases are on the OIE 2014 list of notifiable diseases. This list comprises of transmissible diseases that have the potential for very serious and rapid spread, irrespective of national borders, that are of serious socio-economic or public health consequence and that are of major importance in the international trade of animals and animal product (OIE 2014). Thus the importance of controlling mosquito populations cannot be over-emphasized.

## **2 Materials and methods**

### *2.1 Collection and preparation of plant material*

Collection, drying and storage methods are outlined in a previous publication (Mukandiwa et al. 2013). Dried and powdered leaves of *C. anisata* (500 g) were extracted with acetone (5 L) at room temperature by overnight soaking. The mixture was filtered and the solvent removed under reduced pressure at low temperature (40–50°C) with a rotary evaporator. The extraction process was repeated twice and extracts combined to give 47.87g of dry acetone extract. Part of this extract (23.81 g) was fractionated by solvent-solvent extraction used by

the USA National Cancer Institute (Suffness and Dourous 1979) to yield hexane (4.32 g), chloroform (5.98 g), ethyl acetate (0.05 g), 70% water in methanol (0.25 g) and n-butanol (8.39 g) fractions. The amounts fractionated into the ethyl acetate and the 70% water in methanol fractions were insufficient for further evaluation, thus only the chloroform, hexane and n-butanol fractions were evaluated for activity against mosquitoes. The hexane fraction had the highest activity and was therefore subjected to open column chromatography on silica gel (Kieselgel 60, 70–230 mesh, 0.063–0.200mm, Merck), using the n-hexane: ethyl acetate system at 100:0, 98:2, 95:5, 90:10, 85:15, 80:20 and 70:30(hexane: ethyl acetate). The resulting sub-fractions were combined based on TLC analysis to give 6 sub-fractions. All the extracts and fractions were dissolved in ethanol for the biological assays.

## 2.2 *Test organisms*

*Aedes aegypti* mosquito eggs were obtained from the Pesticide Trial Section of the South African Bureau of Standards (SABS). The eggs were placed in distilled water to hatch. The emerging larvae were fed tropical fish flakes. The emerging adults were fed a 10% sugar solution by placing a soggy cotton wool ball in the cages. Mosquitoes were reared and maintained at  $28 \pm 2$  °C temperature, c.  $45 \pm 10\%$  relative humidity, and a 12:12 (light:dark) photoperiod.

## 2.3 *Repellency assays*

The assays used in this study were approved by the Animal Ethics Committee of the University of Pretoria (Ref: V001-14) in accordance with the national code for the care of animals used in experimentation.

### 2.3.1 Evaluation of *C. anisata* as a topical repellent

Three separate cages each measuring 35 x 35 x 31 cm were used in this assay. For each test material two animals were placed in the same cage every hour for 9 minutes for 3 h. An area of about 80 cm<sup>2</sup> on the back of each animal was shaved and the bare skin areas were cleaned with ethanol. One animal was topically treated with 1 ml aliquot of plant extract on the bare skin area whilst the other was treated with 1 ml of ethanol (negative control). The animals were then, simultaneously placed into a cage with 100 disease-free laboratory-reared mosquitoes. The mosquitoes were starved for 12 hours preceding the test. Before each test, the readiness of the mosquitoes to bite was confirmed by placing an untreated guinea pig into the test cage. Once five mosquito landings and probing attempts were observed on the untreated guinea pig, it was removed from the cage and the test was conducted. The acetone crude extract and the 3 fractions (butanol, chloroform, and hexane) were initially screened for activity at a concentration of 5%. The acetone extract and hexane fraction had the highest activity and the activity was further evaluated at different concentrations. The acetone crude extract was tested at concentrations of 5, 10 and 15% in ethanol, whilst the hexane fraction was tested at 2.5, 5 and 7.5%. The number of landings was recorded over 9 minutes, on an hourly basis, for the treated and control animals before the animals were removed. The number of bites on the animals, identified as pinkish to red tiny bumps, was counted and recorded after 3 hours. A landing is defined as when a mosquito lands on animal for at least 2 seconds without biting. The term bite refers to an insect penetrating skin with its mouthparts and ingesting blood, with resulting abdomen swelling and colour change. Each test was done in triplicate and repeated 3 times with one-week intervals in-between the same treatment and 2-week intervals between different treatments. The same guinea pigs were used and the time intervals allowed for the bitten guinea pigs to heal and also for the extracts effect to wear-off. A commercial formulation that contains 15% DEET served as a positive control in duplicate

experiments. The tests were conducted at a temperature of 25 °C and a relative humidity of c.  $45 \pm 10\%$ .

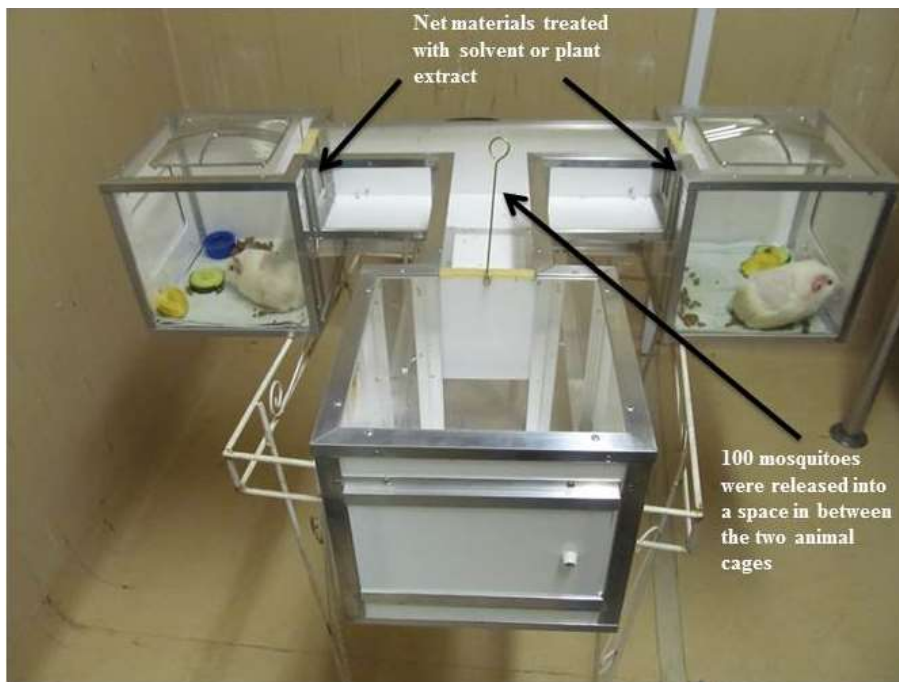
Four of the sub-fractions from the open column chromatography of the hexane fraction were also evaluated at 5% in the same manner as described above. However, the experiments with the subfractions were repeated only twice due to the limited volumes available. The quantities obtained for subfractions 2 and 6 were not enough to conduct biological assays, hence only 4 subfractions were tested.

### 2.3.2 Repellent Treated Nets (Tunnel test design)

The test materials (*C. anisata* acetone extract, the hexane fraction and seselin) were evaluated in an assay that involves the use of guinea pigs as mosquito attractants. Two animals were used to evaluate each test extract concentration. The activity of the extracts was determined at a concentration of 15%. A test device with three cages connected to each other was used (Figure 1). One animal was placed in a cage whose entrance (to the rest of the cage) was fitted with a plant extract treated net. The net, measuring 10 x 9 cm, was treated by applying 3 ml of extract using a pipette and left for 1 hour under open air to allow for the solvent to evaporate before being placed onto the cages. The other animal was placed in a cage whose entrance was fitted with a net treated with ethanol only in the same manner as described for the plant extract and it served as the negative control. One hundred blood-starved mosquitoes were released into a space in between the two animal cages. This method was adapted from the WHOPEs guidelines for efficacy testing of spatial repellents of 2013 (WHO, 2013a). The number of mosquitoes on each side of the cage was counted every 30 minutes for 3 hours. Each test was repeated three times with new mosquitoes. Animals in this assay served solely as attractants but were not directly exposed to the mosquitoes and did not need to be



anaesthetised or restrained. They were supplied with food and water while in the test equipment (Figure 1).



**Fig. 1** Test device with 3 interconnected cages used to test the repellency of *Clausena anisata* extracts when used as net-treating agents with guinea pigs as mosquito attractants.

#### 2.4 Space spraying assays

Testing chambers measuring 27 x 27 x 25.5 cm were used in this evaluation. The plant materials were dissolved in a 1:1 mixture of ethanol and sunflower oil to obtain the desired concentrations. In each test, 1 ml of test materials was nebulised into the chamber using a medical ultrasonic nebuliser which already contained 60 mosquitoes at a rate of 0.2 ml/min. The acetone crude extract and fractions were tested at concentrations of 25, 50, 75, 100 and 150 mg/ml (2.5 - 15%). The ethanol/sunflower oil mixture served as the negative control. The numbers of mosquitoes knocked down at 15, 30 and 60 minutes were counted. The WHO classification of knockdown was used in this study i.e. a knocked-down mosquito is any mosquito that cannot stand; cannot fly in a coordinated manner; cannot take off despite lying

on its back with the legs and wings moving or a mosquito that cannot stay airborne for a prolonged period and repeatedly takes off only to fall down immediately (WHO, 2013b).

In subsequent assays the testing chamber was sprayed with 50 mg/ml (5%) acetone plant extract prior to the introduction of the mosquitoes into the chamber. The mosquitoes were then introduced at 0, 10, 20 and 30 minutes after spraying and the number of mosquitoes knocked down after 0.25, 0.5, 1, and 24 hours was recorded.

### 2.5 *Data analysis*

Results were analysed in SPSS version 22 (IBM). Differences between the means of the test and control group were evaluated using a Student's T-test. Repellency was calculated as:

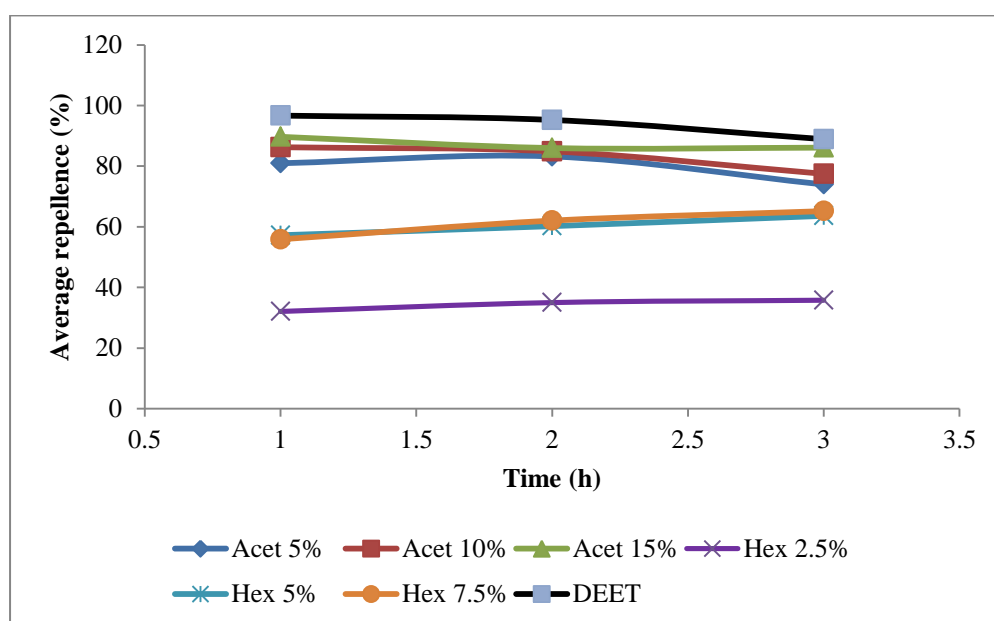
$$\frac{\text{Number of landings/bites on control} - \text{number of landings/bites on treated}}{\text{Number of landings/bites on control}} \times 100$$

The EC<sub>50</sub> values for the adulticidal assay were determined using the pharmacology software Kinetica 5.2 (Thermo Scientific) using non-linear curve fitting for more accurate determination of the EC<sub>50</sub> (Gabrielsson and Weiner 2007).

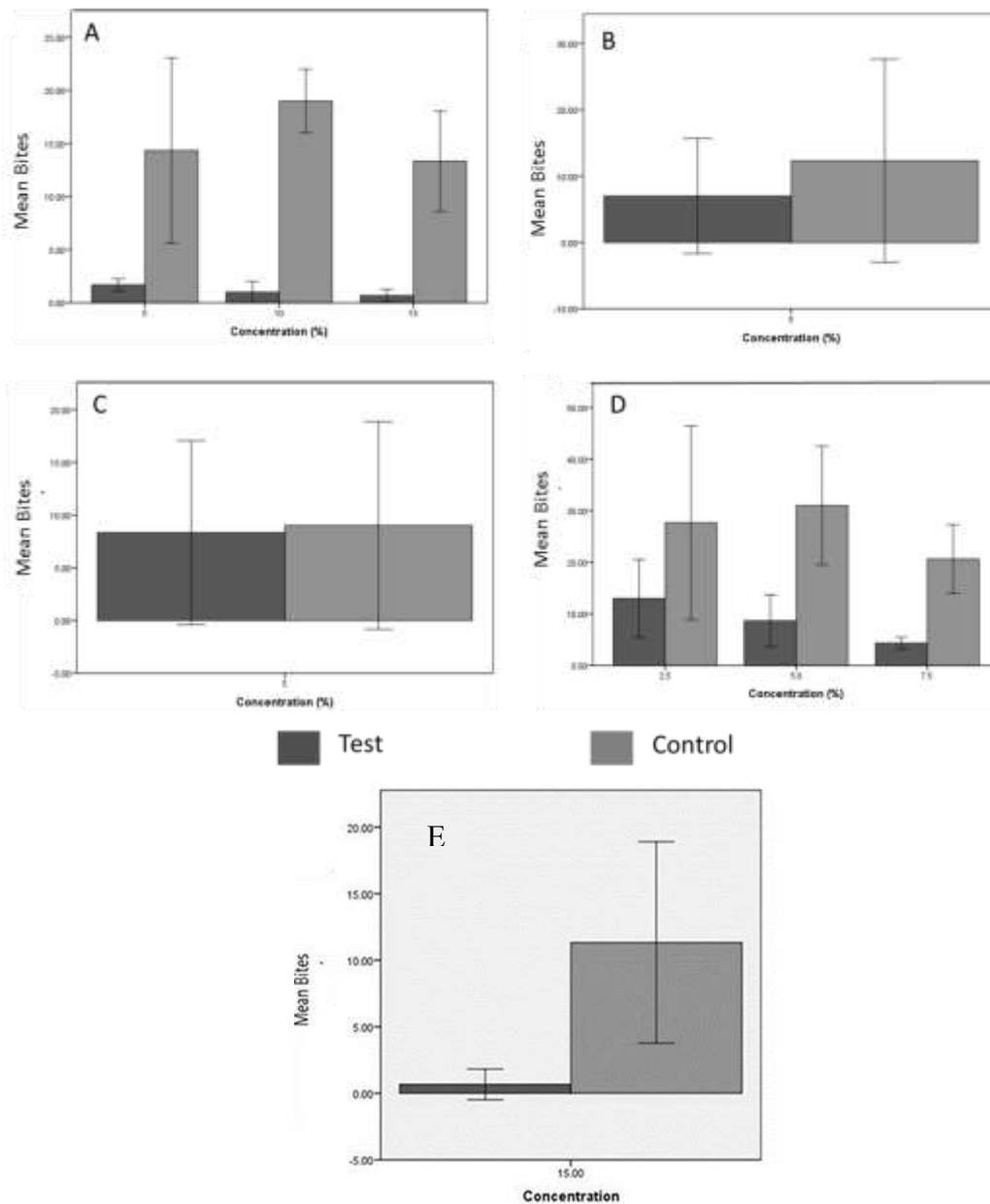
### 3 Results

#### 3.1 Topical application of extracts

Repellency of *C. anisata* extracts against mosquitoes is depicted as both landings and bites (Figures 2 and 3). The average repellency, within 3 hours, measured in landings was relatively lower than in bites for all the tested materials, indicating that the mosquitoes sometimes landed but did not necessarily feed i.e the compound was not effective enough to decrease the attractiveness of feeding, but the effect was sufficient to prevent feeding. The crude extract (15%) had an average repellency, by landings, of 83%, whilst the hexane



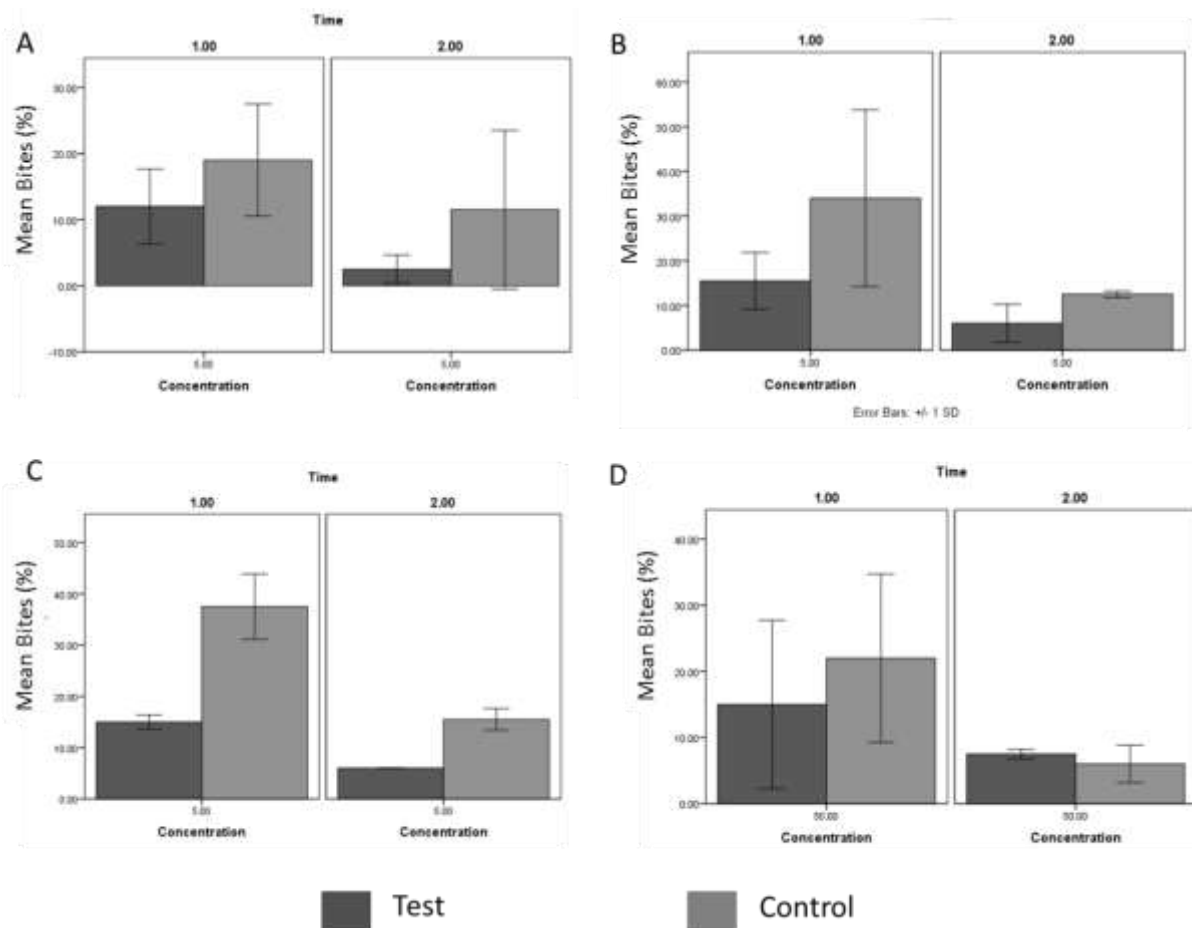
**Fig. 2** The average repellency(%) over time(h) based on the number of mosquitoes landing on guinea pigs treated with different concentration of the crude acetone extract; the hexane fraction and a commercial repellent containing DEET(15%) compared to those landing on the controls (treated with ethanol only)



**Fig. 3** Average repellency of the *Clausena anisata* crude acetone extracts (A); and the butanol (B), chloroform (C) and hexane (D) fractions compared to DEET (E) in terms of the number of mosquito bites on the animal fraction (7.5%) had a repellency of 54%, over the 3 h period. In terms of bites the acetone crude extract (15%) of *C. anisata* had a repellency of 93%, while the hexane fraction (7.5%) of the acetone extract had 67% for up to 3 hours. The numbers of bites on the control and crude extract-treated animals in 9 minutes, in each hour of the study, was significantly different ( $p < 0.05$ ) at all concentrations (Figure 3A). For the hexane fraction, while in general,

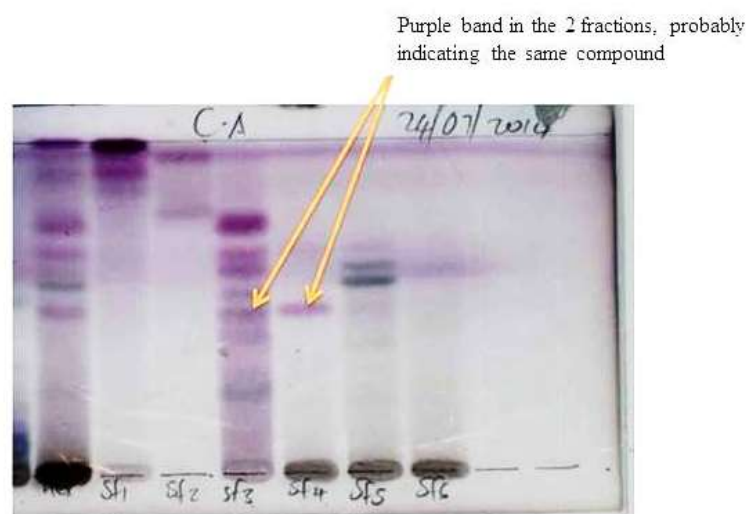
there were more bites on the control group than the treated group, some points were not statistically significant (Figure 3D). DEET (15%), the positive control provided 90% and 100% protection against mosquito landings and bites respectively and differed significantly to the extracts (Figure 3E). The butanol and chloroform fractions were not repellent at the concentrations tested (Figure 3B and C).

The average repellency of each sub-fraction of the hexane fraction, one and two hours after application, is shown in Figure 4. Only sub-fractions 3 and 4 had significant repellency against the mosquitoes, with sub-fraction 4 being superior. On TLC analysis, these 2 sub-



**Fig. 4** Average repellency of the 4 sub-fractions (A-Sub-fraction 1; B-Sub-fraction 3; C-Sub-fraction 4; D-Sub-fraction 5) from *Clausena anisata* leaf extract at 5 %, at one and two hours after application

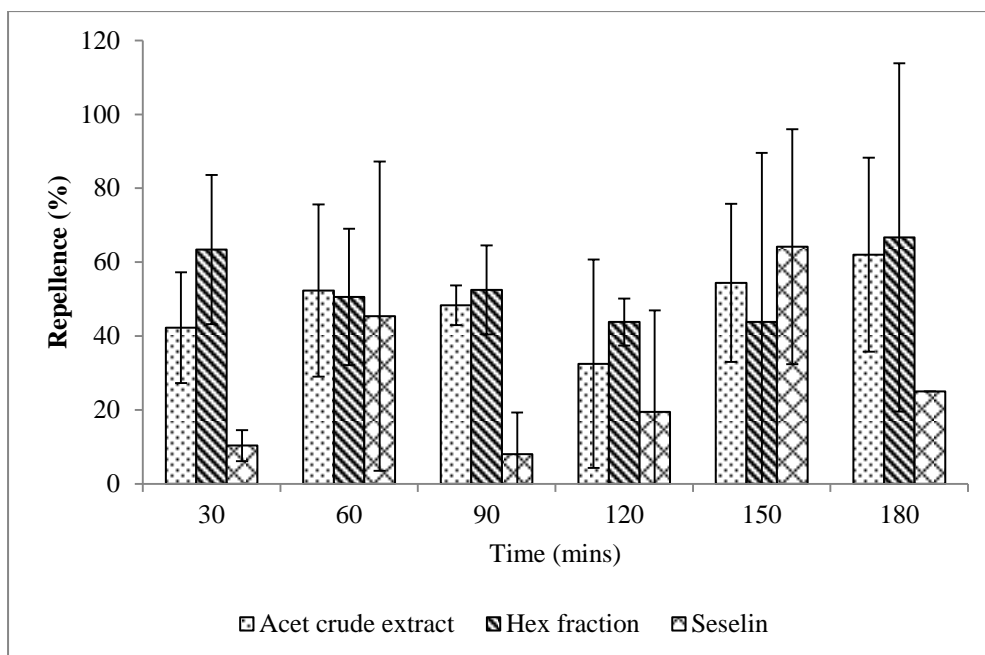
fractions and had one compound in common which was much more concentrated in sub-fraction 4 (Figure 5).



**Fig. 5** Chromatogram of the 6 sub-fractions obtained from the hexane fraction of the *Clausena anisata* extract

### 3.2 Repellence of treated nets

The repellency of the test extracts, when applied on net materials, after every 30 minutes is shown in Figure 6. In general, more mosquitoes were present on the control side than on the sides treated with the leaf acetone crude extract and the hexane fraction of the crude extract. The acetone and hexane extracts of *C. anisata* had average repellences of  $46.89 \pm 2.95$  and  $50.13 \pm 2.02$  %, respectively at 3 h after exposure in the tunnel assay, with no significant temporal variation being present. At ninety minutes post-exposure, an unexpected finding of knock-down became evident with 20% of the mosquitoes dying. The latter effect was observed for both the acetone and hexane extracts. In contrast to the plant extracts, seselin did not repel the mosquitoes with the movement of mosquitoes between the two tunnels showing no clear pattern.

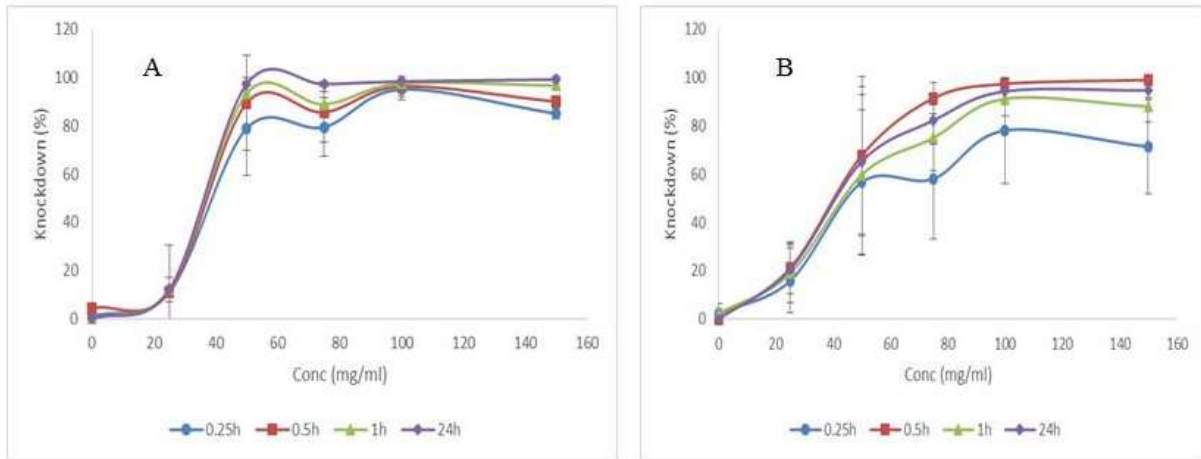


**Fig. 6** Average repellency (%) of the acetone crude extract, hexane fraction and seselin from *Clausena anisata* when applied to net materials over time (mins)

### 3.3 Space spraying assay

The acetone crude extract at concentrations of 5% and above had a significant knockdown effect on mosquitoes at all-time points. The observed difference in knockdown over time, at each concentration point, was not significant (Figure 7A). However the 5 and 7.5% concentrations of crude acetone extract had  $\geq 80\%$  knockdown effect within 30 minutes of spraying, while the 10 and 15% concentrations had greater than 80% knockdown effect within 15 minutes (Figure 7A). A significant difference in the knockdown was observed for the crude extract concentrations  $\leq 2.5\%$  compared to concentrations  $\geq 5\%$  (Figure 7A). The observed differences in knockdown among the crude acetone extract concentrations above 5%, at each time point, were not significantly different. The 5% and above concentrations of the crude acetone extracts caused 24h-mortalities of above 95% (Figure 7A).

For the hexane fraction, there were some significant differences in knockdown ( $P < 0.05$ ) over time at concentrations of 10 and 15% (Figure 7B). Unlike with the crude extract, the



**Fig. 7:** Dose response relationship (% Knockdown) for the acetone crude extract (A) and its hexane fraction (B), at various times to 24 hours

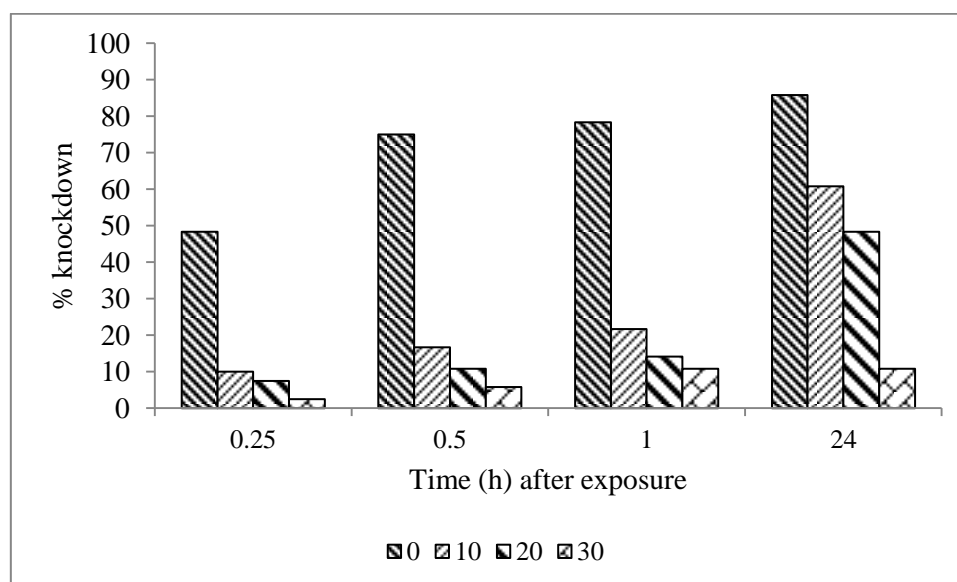
differences in knockdown among the concentrations of the hexane fraction, at each time point, were statistically significant (Figure 7B). None of the hexane extracts had greater than 80% knockdown effect within 15 minutes, with the 10 and 15% hexane fractions concentrations causing over 80% knockdown after 30 minutes. Twenty-four hour mortalities of over 95% were observed at only the concentrations of 10 and 15%. None of the fractions tested were superior that the crude extract. The butanol and chloroform fractions did not have a knockdown effect, even at the highest concentration tested. The  $EC_{50}$  values at each time point of the assay for crude extract and the hexane fraction are given in Table 1, with the highest  $EC_{50}$  value for the crude extract being 78.9 mg/ml (7.89%) at 15 minutes and the lowest being 62.6 mg/ml (6.26%) at 24 hours.



**Table 1: The adulticidal activity (EC<sub>50</sub> (mg/ml)) of the acetone crude extract of *Clausena anisata* and its hexane fraction over different time points**

| Time(h) | Acetone Crude | Hexane Fraction |
|---------|---------------|-----------------|
| 0.25    | 78.9          | 71.7            |
| 0.5     | 66.6          | 128.9           |
| 1       | 66.2          | 109.0           |
| 24      | 62.6          | 107.8           |

For the second phase of the adulticidal assay, in which the mosquitoes were exposed to chambers pre-saturated with the *C. anisata* extract, lower knockdown percentages were observed than when the chambers were saturated with the mosquitoes already present therein. As the interval between spraying the chamber and introduction of mosquitoes into the chamber became longer there was a reduction in mosquito knockdown indicating that saturation within the chamber decreased over time (Figure 8).



**Fig. 8:** Knockdown (%) of adult mosquitoes introduced, at various times (0, 10, 20,30mins), into the testing chamber pre-sprayed with 5% acetone crude extract of *C. anisata*

#### 4 Discussion

In the current study the *C. anisata* acetone extract (15%) and hexane fraction (7.5%) had repellences of 93% and 67% respectively over the 3 hour-testing period. In a previous study by Maharaj et al. (2010) DCM/MeOH leaf extracts (1%) of *C. anisata* had a repellency of 56% against *Anopheles arabiensis* in a 2-minute period. The observed difference in repellency could be attributed to the different solvents used to extract the leaf materials, the different mosquito species used in the assays and the different assay durations. However, at this point, the results of the 2 studies allude that the *C. anisata* plant species certainly contains compounds that are generally repellent to mosquitoes.

Our results compared favourably with the commonly used ingredients of plant-based mosquito repellents on the markets, such as citronella, eucalyptus lemon and *Lippia javanica* oils and para-methane 3-8, diol (PMD) from lemon eucalyptus (*Corymbia citriodora*) extract. Undiluted citronella oil gives 100% protection from host-seeking mosquitoes for about two hours only and loses its activity thereafter (Trongtokit et al. 2005). Another study reports of 20% citronella oil giving 75.7% protection against *A. aegypti* for 2 hours (Amer and Mehlhorn 2006). The ethanolic extract of *Lippia javanica* (5 mg/cm<sup>2</sup>) applied topically provided 100% protection against *A. aegypti* for 8 hours in a laboratory study (Lukwa et al. 2009). PMD applied topically at concentrations ranging from 20% to 50% gives 90 to 100% protection against various mosquito species for time periods ranging from 2h to 12h (Maia and Moore 2011). In comparison, *C. anisata* appears to be a promising candidate as an ingredient of plant-based mosquito repellents.

It is noteworthy that the total number of mosquitoes landing on both the control and treated animals in the assays for DEET and the crude acetone extract was much lower compared to the assay for the hexane fraction. We think that the vaporisation of DEET and crude extract

within the chamber deterred mosquito landings even on the control animal as it was in close proximity with the treated animal. This again, points to the superiority of the crude extract over the fractions, as it is less prone to rapid vaporisation and temporal decrease in effects requiring repeat applications. The deterrence from afar by the acetone extract and the inhibition of biting on those that landed tends to suggest that the mode of action of the extract is both contact and airborne. A number of WHO recommended insecticides against mosquitoes which include the organophosphates, fenitrothion and pirimiphos-methyl, and the carbamates, gendiocarb and propoxur, work in a similar manner (WHO 2013b).

Although seselin, a pyranocoumarin previously isolated from *C. anisata* (Mukandiwa *et al.*, 2013), had larvicidal activity against *A. aegypti* larvae in our previous studies (Mukandiwa *et al.*, 2015); in this study it was not repellent, and it appears that the adulticidal activity observed for the extracts cannot be attributed to seselin. Attempts at isolating a single active component from the plant was not successful as repellency activity decreased as the extract was sequentially separated through fractionation. This tends to suggest that there are various compounds in the crude extract with a synergistic effect that brought out the high efficacy observed in the crude extract. The compound shown in Figure 6 may just be one of them. Similar observations were made by Lukwa *et al.*, (2009) with *Lippia javanica*. Their initial alcohol extract that was made up coumarins, flavanoids and essential oils offered protection against *Aedes aegypti* for 8 h and when the extract was broken down into flavonoids, coumarins and essential oils, all the 3 groups had poor activity giving protection for 2.5 hours at most.

The repellency in the tunnel tests supported the traditional use of the plant whereby the branches of *C. anisata* are hung by the windows and doors to keep mosquitoes away

(Mavundza et al. 2011). With repellency activity not differing significantly over the 3 hours of evaluation, we conclude that the responsible compound(s) are stable and did not vaporise substantially for the extracts to lose their activity over the 3 hour test period. While the compound(s) responsible for the repellence activity are yet to be characterised, the knock-down effect towards the latter part of the test suggests that effect was due to toxicity of the compound to the mosquitoes as naturally insects are repelled by compounds that are potentially toxic to them before they are lethal (Mello and Silva-Filho 2002).

In the space spraying assay the extracts were dissolved in a mixture of ethanol and sunflower oil in an effort to imitate the commercial formulations that comprise of carrier-oils. Examples include pirimiphos-methyl emulsifiable concentrate (EC), an organophosphate, which is dissolved in an oil based solvent and emulsifiers for use in indoor residual spraying (IRS) as recommended by WHO (2013b). The increased mosquito knockdown with increasing concentration of *C. anisata* strongly indicates that the observed knockdown is a biological effect of compounds within the extracts on the mosquitoes. The high incidence of knockdown obtained in the first 15 minutes after spraying the 10 and 15 % crude extract make the extract a promising candidate for development into a mosquito aerosol spray. The superiority of the crude extract was again evident in this assay, with an EC<sub>50</sub> value of 7.89% and 6.26% at 15 minutes and 24 hours after spraying. The adulticidal activity of *C. anisata* against a different mosquito species, *Anopheles arabiensis*, albeit low levels, has been reported by Mavundza et al. (2014). In their study they exposed the adult mosquitoes to filter paper impregnated with the plant extract in a WHO test kit. It is noteworthy that this low activity was observed at a *C. anisata* extract concentration of 1%. Although the adulticidal activity reported may differ between our study and that of previous researchers, this may be due to a number of reasons such as type of assay and extracts used; results from both studies

again suggest that *C. anisata* leaves are a potential source of compounds with mosquito adulticidal activity.

## **5 Conclusion**

*Clausena anisata* extracts have potential use as ingredients in protection products against mosquitoes. This may in part be because of the repellent and adulticidal compounds that they contain. By examining the activity of different fractions of the crude extract we could show that the activity must be based on some synergistic activity between different compounds. This could lead to reduced development of resistance. The crude extract also had both contact and airborne activities. These are highly regarded activities for WHO approved pesticides. Furthermore the crude extract is very easy to prepare and could lead to a commercial product. At this stage, we believe that the product may have merit for further design for application at nights, either as a lotion or as a soap. In addition this study validates the use of *C anisata* in ethnomedicinal practice. This is an important finding, in terms of disease mitigation, as ineffective products can lead to an increase in the prevalence of the disease in household that still rely on this plant species for protection from mosquitoes. Further work should be directed towards the standardisation of the *C. anisata* extracts and fractions. We found that there was a loss in activity in our attempts to isolate the active compound.

## **6 Competing Interests**

The authors declare that they have no competing interests

## 7 *Authors' contributions*

LM designed and conducted the experiments and drafted the manuscript. VN participated in the design of the study, data analysis and interpretation of the results. JNE was involved in originating the research and was involved in writing up the manuscript. All authors read and approved the final manuscript.

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